DEVELOPMENT OF LUCIFERASE TAGGED ANTHRAX BACTERIA TO EVALUATE NOVEL THERAPIES USING IN VIVO IMAGING

Rina Das, Sachin Mani, Maria Mayda, Rebecca Gurba and Marti Jett
Division of Pathology, Walter Reed Army Institute of Research, Silver Spring, MD 20910.

ABSTRACT

We have developed a quantitative imaging tool of bioluminescence to visualize the anthrax infection process in real time. This involves transfection of luciferase gene that produces photons that are captured by a highly sensitive CCD camera using the IVIS Imager from Xenogen. We have transfected B. anthracis (Sterne) with the pXEN5 plasmid that contains the lux operon, kanamycin resistant colonies were selected that showed luminescent signals. In vitro assays on mouse macrophage cell line has been used to determine the infectivity of the transfectants. These luminescent transfectants will then be evaluated in an animal model. This study will also enable us to evaluate the efficacy of nanoparticles encapsulated antibodies/drugs for therapy.

INTRODUCTION

The recent exposures to *Bacillus anthracis* have highlighted certain issues that are unclear, especially the time frame for which spores could reside in the lungs, and their potential to subsequently cause a lethal outcome. It is important to find a technique that will allow for visual monitoring of the entire infection process in real time to get a better understanding of the sequence of events and target tissues affected by this bacteria.

Methods for optically monitoring infectious events in vivo that involve an external light source may be limited by tissue-intrinsic background noise, even with fluorescent markers. The advantage of bioluminescence is the absence of background light from mammalian cells. Imaging bioluminescence as an indicator of biological processes in animals can be used to address a number of issues in the study of pathogenesis, as well as efficacy for therapy and vaccine regimens.

This technology is different than measuring fluorescence since there is no need to have an external light source for excitation and then look for the emission. Use of luciferase reporter genes that are integrated into the genome and whose detectable signal is inextricably linked to the metabolic activity of the target cell population, produces a bioluminescent signal that can be captured by sensitive CCD cameras. Optimal reporter genes for in vivo cell trafficking studies should encode well-characterized gene products with deeply penetrating emission and a high signal-to-noise ratio.

Contag et al. (1995) reported the development of a method capable of detecting and monitoring bioluminescent bacteria within a living host by using an intensified charge-coupled device (ICCD) camera. Therefore, newly developed technology now makes it possible to track the location or effects of biological warfare (BW) agents in vivo in animals and to extend these studies over any time course desired while still keeping the animal alive (Fig 5). This is in vivid contrast with the early studies of localization whereby numerous animals would be sacrificed at each time point and laborious methods employed to recover a “labeled” BW agent.

Our approach was to optically monitor the infectious event of anthrax and evaluate efficacy of novel therapies in a live mouse model by using the new technology of bioluminescence and in vivo imaging. Host pathogen interactions triggered by an infection will be monitored in real time by a non-invasive imaging method. The utility of in vivo imaging, of a

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01 OCT 2005	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Development Of Luciferase Tagged Anthrax Bacteria To Evaluate Novel Therapies Using In Vivo Imaging	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Division of Pathology, Walter Reed Army Institute of Research, Silver Spring, MD 20910.**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
See also ADM001851, Proceedings of the 2003 Joint Service Scientific Conference on Chemical & Biological Defense Research, 17-20 November 2003. , The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**7**	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18

diseased animal has tremendous potential for testing therapeutic drugs or vaccines for their efficacy in an established animal model.

The second objective of the study is to design and test novel therapies for anthrax using the in vivo imaging technique. Traditional approach for treatment of anthrax is using antibiotics, since the window of opportunity for such treatment is so small that we wanted to explore other options of treatment at later time periods of the infection process. B. anthracis can be targeted by using antibodies specific for the spore protein, protective antigen or lethal factor that have shown immune responses in animals. Our eventual aim is to develop novel therapies for targeting anthrax. These could include removing the pathogen from the host by using biocompatible nanoparticles to a) entrap spores, b) deliver antisera directed against either the protective antigen (PA) or lethal factor (LF) or c) extend the half-life of phage lytic enzymes directed against B. anthracis spores/vegetative cells.

The present study deals with the design and development of luciferase tagged *Bacillus anthracis* (Sterne) to create luminescent bacteria for real time visualization and monitoring of the infection process by in vivo imaging in a rodent animal model.

MATERIAL AND METHODS

Bacterial strains, plasmid and culture media: The bacterial strain and plasmids used in this study are Bacillus anthracis (sterne) strain, E.coli from Invitrogen, pXen5 plasmid was obtained from Xenogen, Inc.

The growth media used for cultivating B. anthracis were Brain Heart Infusion (BHI) from DIFCO, as well as BYGT (BHI, yeast extract, glucose, glycerol, and Tris pH8) as described by Dunny et al. (1981). *E. coli* was cultured in LB medium. Antibiotics used in selective media were Erythromycin at 2μg/ml and kanamycin from 1 to 20μg/ml for *B. anthracis*.

Sporulation was induced using Modified Schaeffer's Sporulation broth consisting of Difco Nutrient Broth, Potassium chloride, Magnesium Sulfate, Calcium Chloride, Ferrous sulfate monohydrate, Manganese chloride and Glucose as described in Leighton and Doi (1971).

Transformation and selection of E. coli and B anthracis: Plasmid DNA (pXen-5- Figure1) was isolated from *E. coli* using a plasmid isolation kit (Quiagen). The transformation method used in this experiment was adopted from Dunny et al. (1991) with minor modifications. For *B. anthracis* transformation, the bacteria were grown in BYGT medium to an optical density at 590 of 0.2 to 0.4 absorbance units. After chilling the cells on ice in electroporation buffer (0.625 M sucrose, 1mM $MgCl_2$ adjusted to pH 4.0) cells were electroporated using 2ug of plasmid pXen-5 in 0.2-cm cuvette with 25μF setting on the Gene Pulse, a resistance of 200 Ω and the field strength set at 8.7 to 10 kV/cm. The cells were first incubated for 90 min. at 30^{o}C, after that were plated on selective BHI or LB media with antibiotics and incubated for 24 to 48 hr.

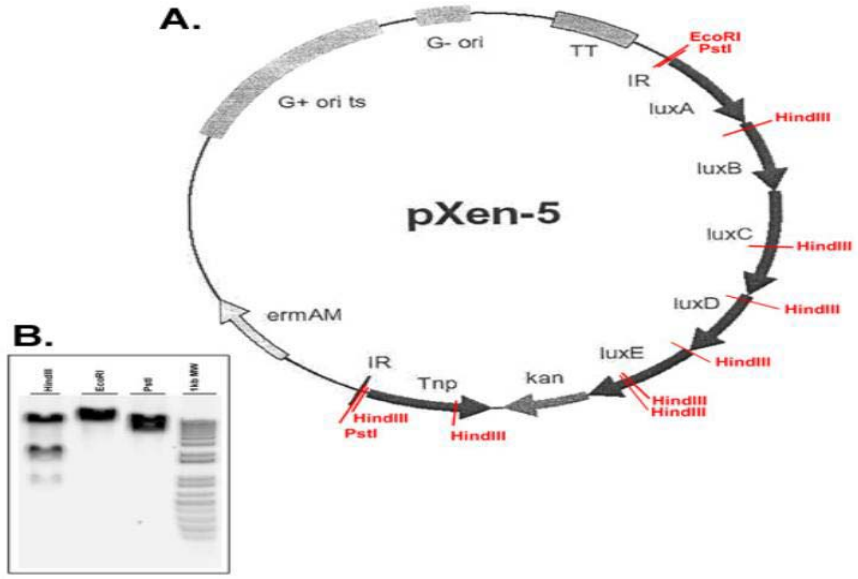


Figure 1. Identity verification of the plasmid pXen-5 isolated from *E. coli* through restriction digestion. (**A**) The restriction map of plasmid pXen-5. (**B**) Hind III, EcoRI, and PstI digests of plasmid pXen-5 along with a 1kb molecular weight marker on an agarose gel.

Screening for stable, highly bioluminescent *B anthracis Sterne:* Transformants of *B. anthracis* containing pXEN-5 were first patched onto LB-agar plates containing erythromycin (2µg/ml) and incubated overnight at 30°C. Erythromycin resistant colonies were then selected over different concentrations of LB-Kanamycin (up to 20µg/ml) at 42°C. The resulting colonies were then screened for bioluminescence signal using an ICCD camera and the brightest colonies were streaked onto LB agar plates containing kanamycin (1µg/ml). Single colonies were streaked subsequent times onto LB agar plates containing no antibiotic to verify that bioluminescence was stable in the absence of antibiotic selection. Each clone was then graded for its level of bioluminescence using an ICCD camera and Xenogen's LivingImage software (Xenogen Corporation, Alameda, Calif.)

Induction of spores: A single colony of B. anthracis was grown overnight in BHI broth at 37° C. One loop full from the broth culture was streaked on BHI agar and incubated at 37° C overnight. The colony was used to inoculate a 500 ml flask with 20 ml of Modified Schaeffer's Sporulation Broth (2XSG). 10 ml of the cultures was transferred into 1-liter flasks with 200 ml of fresh 2XSG broth. After the overnight incubation, the flask was removed and set aside at room temperature without disturbance for 10 days, and spores were induced according to Leighton and Doi (1971).

In-vitro macrophage cell assay for spore infection: J774A macrophage cells were used to check for phagocytosis after exposure to the spores. Cells were suspended in Dubelcco's Minimal Essential Medium (DMEM) with fetal bovine serum (FBS, Gibco BRL) and adjusted to 10,000 cells/ml and incubated with 3 to 50 MOI of spores. After 30 minutes the cells were washed and plated in 96-well flat-bottom plates. Media was removed and the plates were frozen after 24, 48 and 72 hours and the viability was measured using the CYQUANT kit (Molecular Probes) according to manufacturer's protocol and compared to the untreated control cells. Each treatment was performed in replicates of six and each experiment was repeated three times.

RESULTS AND DISCUSSION

Real time imaging is made possible by the use of bioluminescent bacteria and a powerful IVIS imager with a CCD camera. For the present study we transformed B anthracis Sterne strain with the luciferase genes. The plasmid pXen-5 was used for transformation, which contains all the lux genes, erythromycin and kanamycin antibiotic resistance genes and the transposon that allows for chromosomal integration. Colonies resistant to kanamycin were selected for stable transfectants.

Optimization of electroporation and growth conditions for B. anthracis. B anthracis was transformed by electroporationas as shown previously (1, 4). Following the first transformation experiment using 0.5µg of plasmid DNA, no erythromycin resistant colonies were observed. However, both 1 and 2µg of plasmid DNA resulted in similar amounts of resistant colony forming units per plate (CFU/ plate) (Table 1). Control plates containing wild type B. anthracis without the pXen-5 plasmid failed to grow on erythromycin or kanamycin agar plates. No apparent differences were observed in average CFU/ plate between 2.0 and 2.5 Volts used for electroporation.

Table 1. Colony Forming Units that grew on LB agar plates with 2µg/ml erythromycin under the specified parameters.

2.0 Volts		2.5 Volts	
Plasmid (µg)	CFU	Plasmid (µg)	CFU
0.5	0	0.5µg DNA	0
1.0	35	1.0µg DNA	48
2.0	40	2.0µg DNA	39

When the optical density at 590nm was in the range of 0.35 to 0.4 absorbance units for bacteria prior to preparation for competent cells, maximum transformants were obtained. From 6 to 20 trasformants/µgDNA was obtained using the 18kb pXen-5 plasmid.

Selection of B. anthracis with chromosomal integration:

Forty colonies were chosen from the erythromycin resistant bacteria and grown onto LB agar plates with 1, 5, 10, 15, and 20µg/ml of kanamycin. Of the colonies plated, 27.5% showed signs of growth at different concentrations (Fig. 2). Only 12.5% colonies were able to survive at 20µg/ml of kanamycin. Although increasing the concentration of kanamycin in the medium gave rise to fewer transformants, the proportion of colonies producing higher levels of bioluminescence was found to increase. This indicates that increasing the concentration of kanamycin in the medium results in the selection of integration sites with stronger promoters upstream of the *luxABCDE* km^r operon (Figure 3).

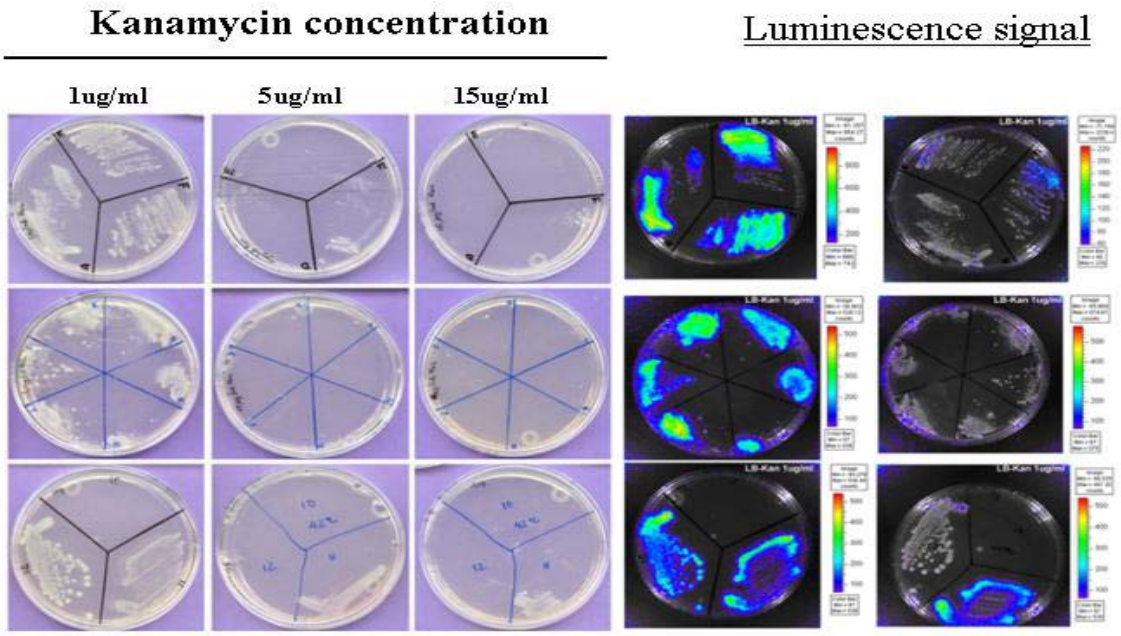


Figure 2. Kanamycin resistant colonies of B anthracis. Colonies initially grown on LB erythromycin were re-plated onto LB plates with different concentrations of Kanamycin.

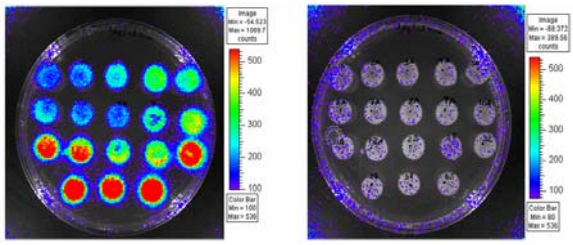


Figure 3. Luminescent colonies of B anthracis grown in agar plates with kanamycin showing different luciferase activity as measured by the Xenogen's IVIS imager. Blue color shows less luminescent compared to red signal, which depict higher light signal.

Promoter strength can be selected by varying the kanamycin concentration in the medium during isolation of chromosomal integrants. As we can see in Figure 2 transformants that show growth on high concentrations of kanamycin, are more luminescent.

Macrophage assay for spore infection: The survival of macrophages after infection with B anthracis spores was determined by measurement of viable cell number using the CYQUANT method to measure total DNA fluorescence. To determine the ideal MOI, J774-A cells were exposed to different MOI of spores. A 40% reduction was observed at 10 MOI, which was used in a time course experiment. As is shown in figure 4, the macrophage cell assay was sensitive to infection with B anthracis spores at various MOI.

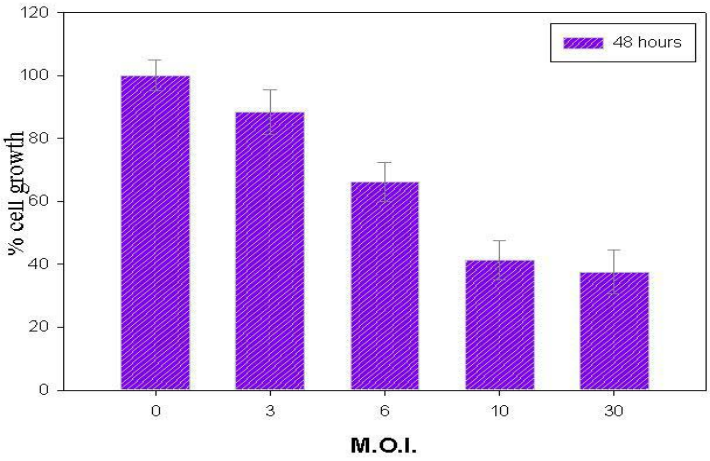


Figure 4A

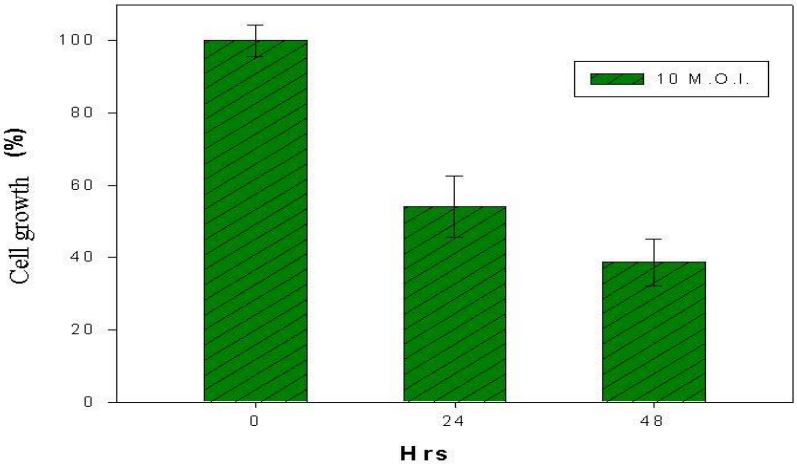
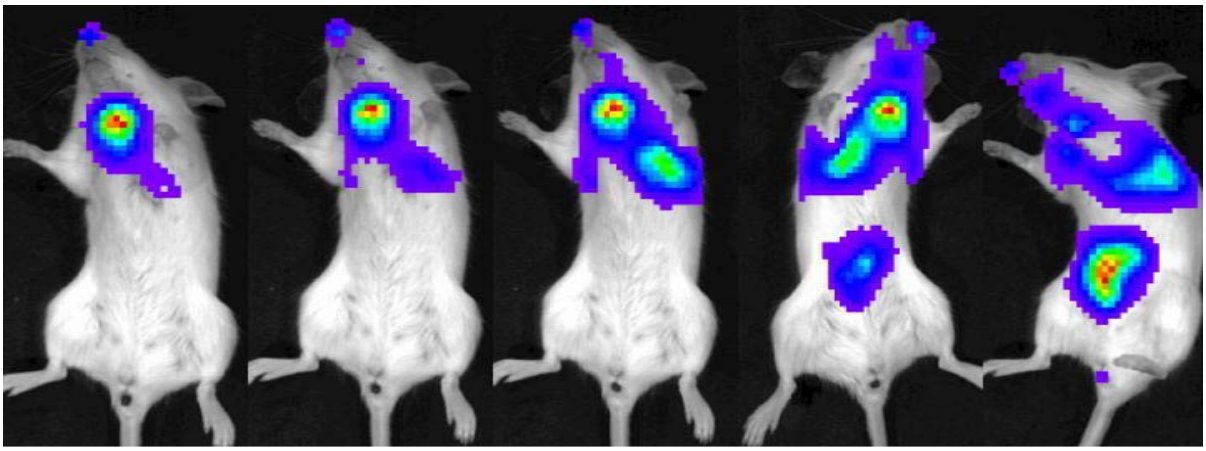


Figure 4B

Figure 4A. Macrophage cell survival assay: Cells were incubated with different MOI of B anthracis spores for 48hrs and cell number measured. Figure 4B. Shows cell survival at 24h and 48h after using 10 MOI of spores for infection.

In vitro macrophage cell culture system is used to test the efficacy of anthrax specific antibodies coated nanoparticles in scavenging or preventing an infection of *Bacillus anthracis* (spores or vegetative forms).

Studies of the infectious process and antimicrobial efficacy in vivo have typically involved introduction of the infectious agent, antibiotic treatment, and eventual quantitation of the bacteria ex vivo from various sites within the host animal. As shown in figure 5 we have infected a BALB C mouse with Yersinia pseudotuberculosis and the images of the same animal captured by the IVIS Imager (Xenogen). Without sacrificing the animal one can show the progression of the disease in real time using this in vivo imaging technology. Similar studies will be performed using luminescent B. anthracis.



Time course of bioluminescent *Yersinia pseudotuberculosis* infection in real time in a BALB/c mouse model using in vivo imaging

Figure 5: In vivo imaging of a live animal with luminescent bacteria after intratracheal infection.

Ideally, studies allowing noninvasive monitoring of the bacterial infection in vivo, using a bioluminescent reporter system permits assessment of the disease process and allows monitoring of the same animal throughout the duration of study. Such an approach might provide more information during infection studies, imparting more statistical power while using fewer animals.

CONCLUSION

The luciferase gene was inserted into the chromosome of B anthracis sterne strain and stable transfectants obtained. One hundred kanamycin-resistant B. anthracis colonies were tested for luminescence signal and five stable tranfectants were selected using the IVIS imager. These luminescent anthrax bacteria will be used in an animal model for in vivo imaging to visualize the infection process in real time and test the efficacy of various novel therapeutic molecules. Macrophage cell assay has been established for screening of therapeutic agents such as nanoparticle-encapsulated antibodies to anthrax for combating the disease even at later stages of infection.

REFERENCES

1. Contag, C. H., P. R. Contag, J. I. Mullins, S. D. Spilman, D. K. Stevenson, and D. A. Benaron. 1995. Photonic detection of bacterial pathogens in living hosts. Mol. Microbiol.18: 593-603.

2. Dunny, G. M., C. Funk, and J. Adsit. 1981. Direct stimulation of the transfer of antibiotic resistance by sex pheromones in Streptococcus faecalis. Plasmid 2:270-278.

3. Dunny, G., M., N. Lee, and D. J. LeBlanc. 1991. Improved electroporation and cloning vector system for gram-positive bacteria. Applied and Environmental Microbiology, Apr. 1991: 1194-1201.

4. Francis, K. P., J. Yu, C. Bellinger-Kawahara, D. Joh, M. J. Hawkinson, G. Xiao, T. F. Purchio, M. G. Caparon, M. Lipsitch, and P. R. Contig. 2001. Visualizing Pneumococcal infections in the lungs of live mice using bioluminescent Streptoccus pneumoniae transformed with a novel gram-positive lux transposon. Infection and Immunity 69, No. 5: 3350-3358.

5. Leighton, T. J, R.H. Doi. 1971. The stability of messenger ribonucleic acid during sporulation in Bacillus subtilis. Journal of Biological Chemistry 246: 3189-95.